HPLC

A Practical User's Guide

SECOND EDITION

Marvin C. McMaster

WILEY-INTERSCIENCE
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HPLC

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PREFACE

High-pressure liquid-solid chromatography (HPLC) is rapidly becoming the method of choice for separations and analysis in many fields. Almost anything that can be dissolved can be separated on some type of HPLC column. However, with this versatility comes the necessity to think about the separation desired and the best way to achieve it. HPLC is not now and probably never will be a turn-key, push-button type of operation. Many dedicated system-in-a-box packages are sold for specific separations, but all of these still offer wide possibilities for separation. Changing the column and the flow rate lets you change the separation and the amount of sample you can inject. This is not the worst thing in the world, for it does create great opportunity for the chromatographer and a great deal of job security for the instrument operator.

Fortunately, controlling separations is not nearly as complicated as much of the literature may make it seem. My aim is to cut through much of the detail and theory to make this a usable technique for you. The separation models I present are those that have proven useful to me in predicting separations. I make no claim for their accuracy, except that they work. There are many excellent texts on the market, in the technical literature, and on the Internet, continuously updated and revised, that present the history and the current theory of chromatography separations.

This book was written to fill a need, hopefully, your need. It was designed to help the beginning as well as the experienced chromatographer in using an HPLC system as a tool. Twenty-five years in HPLC, first as a user, then in field sales and application support for HPLC manufacturers, and finally working as a teacher and consultant has shown me that the average user wants an instrument that will solve problems, not create new ones.

I will be sharing with you my experience gained through using my own instrument, through troubleshooting customer's separations, and from field demos; the tricks of the trade. I hope they will help you do better, more rapid separations and methods development. Many of the suggestions are based on tips and ideas from friends and customers. I apologize for not giving them credit, but the list is long and my memory is short. It has been said that plagiarism is stealing ideas from one person and research is borrowing from many. This book has been heavily researched and I would like to thank the many

who have helped with that research. I hope I have returned more than I borrowed.

I have divided this guide into three parts. The first part should give you enough information to get your system up and running. When you have finished reading it, put the book down and shoot some samples. You know enough now to use the instruments without hurting them or yourself. When you have your feet wet (not literally I hope), come back and we will take another run at the material in the book.

Part II shows you how to make the best use of the common columns and how to keep them up and running. (Chapter 6 on column healing should pay for the book in itself.) It discusses the various pieces of HPLC equipment, how they go together to form systems, and how to systematically troubleshoot system problems. We will take a look at the newest innovations and improvements in column technology and how to put these to work in your research. New detectors are emerging to make possible analysis of compounds and quantities that previously were not detectable.

Finally, in Part III, we will talk about putting the system to work on real-world applications. We will look at systematic methods development, both manual and automated, and the logic behind many of the separations that others have made. We will discuss how to interface the HPLC system to computers and robotic workstations. I will also give you my best guesses as to the direction in which HPLC columns, systems, detectors, and liquid chromatography/mass spectrometer (LC/MS) systems will be going.

It is important to give credit where it is due. Christopher Alan McMaster created many of the illustrations in this text before he died of the ravages of muscular dystrophy six years ago. I supplied hand-drawn sketches of the illustrations I used on boards in my classes. Chris turned them into art on his Macintosh. His collaborative efforts are greatly missed.

A brief note is required about the way I teach. First, I have learned that repetition is a powerful tool, not a sign of incipient senility as many people have hinted. Second, I have found in lecturing that few people can stand more than 45 minutes of technical material at one sitting. However, I have also learned that carefully applied humor can sometimes act as a mental change of pace. Properly applied, it allows us to continue with the work at hand. So, occasionally, I will tiptoe around the lab bench. I do not apologize for it, but I thought you ought to know.

The instrument itself is the most effective teacher. Think logically about the system and the chemistry and physics occurring inside the column. You will be surprised how well you will be able to predict and control your separation.

Remember! HPLC is a versatile, powerful, but basically simple separation tool. It is a time machine that can speed your research and, thereby, allow you to do many things not possible with slower techniques. It is both an analytical and a preparative machine. When I finish, I hope you will have the confidence to run your instrument, make your own mistakes, and be able to find your own solutions.

Your HPLC success depends on three things:

1. The suitability of the equipment you buy,
2. Your ability to keep it up and running (or find someone to service it), and
3. The support you receive, starting out in new directions or in solving problems that come up.

Marvin C. McMaster
Florissant, MO

I

HPLC PRIMER

1

ADVANTAGES AND DISADVANTAGES OF HPLC

The first things we need to understand are how an HPLC system works, its best applications and advantages over other ways of separating compounds, and other techniques that might compliment or even replace it. Is there a faster, easier, cheaper, or more sensitive method of achieving your results? The answer is yes, no, maybe. It really depends on what you are trying to achieve.

HPLC's virtue lies in its versatility! I have used it to separate compounds of molecular weights from 54 to 450,000 Daltons. Amounts of material to be detected can vary from picograms and nanograms (analytical scale) to micrograms and milligrams (semi-preparative scale) to multigrams (preparative scale). There are no requirements for volatile compounds or derivatives. Aqueous samples can be run directly after a simple filtration. Compounds with a wide polarity range can be analyzed in a single run. Thermally labile compounds can be run. I had one customer whose company made explosives for primers. Her first job of the day was to explode samples of the previous day run with a rifle. Her second job was to carry out an HPLC analysis of that day's run.

An HPLC offers a combination of speed, reproducibility, and sensitivity. Typical runs take from 10 to 30 min, but long gradients might consume 1 to 2 hrs. I have seen 15- to 30-sec stat runs on 3-μm columns in hospital laboratories. Retention times on the same column, run to run, should reproduce by 1%. Two columns of the same type from the same manufacturer should give 5% or better retention time reproduction on the same standard set.

While the HPLC can be used in a variety of research and production operations, there are a few places where it really shines. Because it can run

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underivatized mixtures, it is a great tool for separating and analyzing crude mixtures with minimum sample preparation. I began my HPLC career analyzing herbicide production runs as a method of trouble-shooting product yield problems. HPLC was routinely used in the quality control lab to fingerprint batches of final product using a similar analysis. I have helped my customers run tissue extracts, agricultural run-off waters, urine, and blood samples with minimum clean up. These samples obviously are not very good for columns whose performance degrades rapidly under these conditions. Columns can usually be restored with vigorous washing, but an ounce of prevention is generally more effective than a pound of cure and also much more time effective.

Standards purification is another role in which the HPLC excels. It is fairly easy to purify microgram to milligram quantities of standard compounds using the typical laboratory system.

Finally, used correctly, HPLC is a great tool for rapid reaction monitoring either in glassware or in large production kettles. I started my analytical career with a HPLC system cast-off by the Analytical Department and a 15-min training course by another plant monitoring chemist. He gave me an existing HPLC procedure for my compound and turned me loose. The next day I was getting research information. I could see starting material disappear, intermediates form, and both final product and by-products appear. It was like having a window on my reaction flask through which I could observe the chemistry of the ongoing synthesis. Later, I used the same technique to monitor a production run in a 6000-gallon reactor. The sampling technique was different, but the HPLC analysis was identical.

Versatility, however, brings with it challenge. An HPLC is easily assembled and easily run, but to achieve optimum separation, the operator needs to understand the system, its columns, and the chemistry of the compounds being separated. This will require a little work and a little thought, but the skills required do offer a certain job security.

I don't want to leave you with the impression that I feel that HPLC is the perfect analytical system. The basic system is rather expensive compared with some analytical tools; columns are expensive with a relatively short operating life, solvents are expensive and disposal of used solvent is becoming a real headache. Other techniques offer more sensitivity of detection or improved separation for certain types of compounds (i.e., volatiles by GLC, large charged molecules by electrophoresis). Nothing else that I know of, however, offers the laboratory the wide range of separating modes, the combination of qualitative and quantitative separation, and the basic versatility of the HPLC system.

1.1 HOW IT WORKS

The HPLC separation is achieved by injecting the sample dissolved in solvent into a stream of solvent being pumped into a column packed with a solid sep-

arating material. The interaction is a liquid-solid separation. It occurs when a mixture of compounds dissolved in a solvent can either stay in the solvent or adhere to the packing material in the column. The choice is not a simple one since compounds have an affinity for both the solvent and the packing.

On a reverse-phase column, separation occurs because each compound has different partition rates between the solvent and the packing material. Left alone, each compound would reach its own equilibrium concentration in the solvent and on the solid support. However, we upset conditions by pumping fresh solvent down the column. The result is that components with the highest affinity for the column packing stick the longest and wash out last. This differential washout or elution of compounds is the basis for the HPLC separation. The separated, or partially separated, discs of each component dissolved in solvent move down the column, slowly moving farther apart, and elute in turn from the column into the detector flow cell. These separated compounds appear in the detector as peaks that rise and fall when the detector signal is sent to a recorder or computer. This peak data can be used either to quantitate, with standard calibration, the amounts of each material present or to control the collection of purified material in a fraction collector.

1.1.1 A Separation Model of the Column

Since the real work in an HPLC system occurs in the column, it has been called the heart of the system. The typical column is a heavy-walled stainless steel tube (25-cm long with a 3–5 mm i.d.) equipped with large column compression fittings at either end (Fig. 1.1).

Immediately adjacent to the end of the column, held in place by the column fittings, is a porous, stainless steel disc filter called a frit. The frit serves two purposes. It keeps injection sample particulate matter above a certain size from entering the packed column bed. At the outlet end of the column it also serves as a bed support to keep the column material from being pumped into the tubing connecting out to the detector flow cell. Each column end fitting is drilled out to accept a zero dead volume compression fitting, which allows the column to be connected to tubing coming from the injector and going out to the detector.

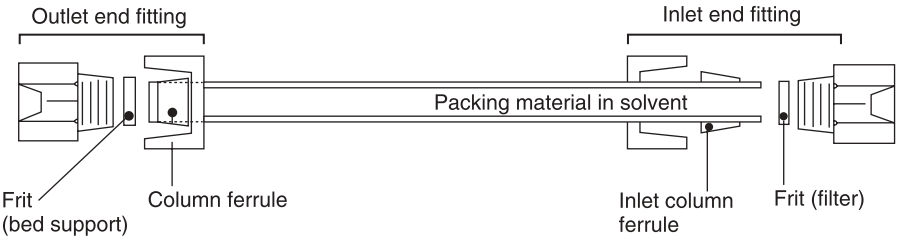

Figure 1.1 HPLC column design.

The most common HPLC separation mode is based on separating by differences in compound polarity. A good model for this partition, familiar to most first-year chemistry students, is the separation that takes place in a separatory funnel using immiscible liquids such as water and hexane. The water (very polar) has an affinity for polar compounds. The lighter hexane (very nonpolar) separates from the water and rises to the top in the separating funnel as a distinct upper layer. If you now add a purple dye made up of two components, a polar red compound and a nonpolar blue compound, and stopper and shake up the contents of the funnel, a separation will be achieved (Fig. 1.2).

The polar solvent attracts the more polar red compound, forming a red lower layer. The blue nonpolar dye is excluded from the polar phase and dissolves in the relatively nonpolar upper hexane layer. To finish the separation, we simply remove the stopper, open the separatory funnel's stopcock, and draw off the aqueous layer containing the red dye, and evaporate the solvent. The blue dye can be recovered in turn by drawing off the hexane layer.

The problem with working with separatory funnels is that the separation is generally not complete. Each component has an equilibration concentration in each layer. If we were to draw off the bottom layer and dry it to recover the red dye, we would find it still contaminated with the other component, the blue dye. Repeated washings with fresh lower layer would eventually leave only insignificant amounts of contaminating red dye in the top layer, but would also remove part of the desired blue compound. Obviously, we need a better technique to achieve a complete separation.

The HPLC column operates in a similar fashion. The principle of "like attracting like" still holds. In this case, our nonpolar layer happens to be a moist, very fine, bonded-phase solid packing material tightly packed in the column. Polar solvent pumped through the column, our "mobile phase," serves as the second immiscible phase. If we dissolve our purple dye in the mobile phase, then inject the solution into the flow from the pump to the column, our two compounds will again partition between the two phases. The more non-

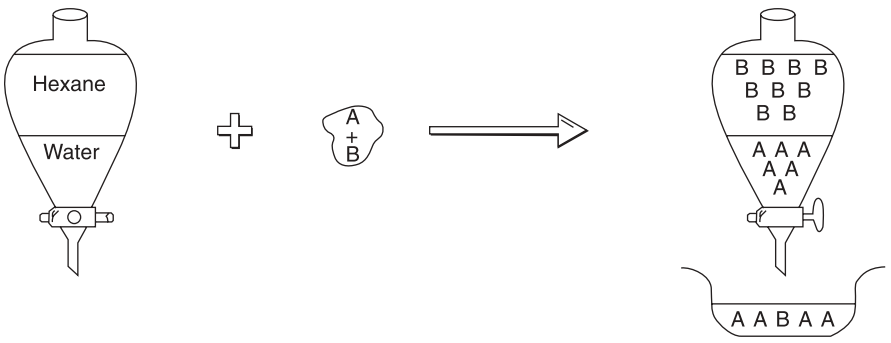

Figure 1.2 Separation model 1 (separatory funnel).

polar blue dye will have a stronger partition affinity for the stationary phase. The more polar red dye favors the mobile phase, moves more rapidly down the column than the blue dye, and emerges first from the column into the detector. If we could see into the column we would see a purple disc move down the column, gradually separating into a fast moving red disc followed by a slower moving blue disc (Fig. 1.3).

1.1.2 Basic Hardware: A Quick, First Look

The simplest HPLC system is made up of a high-pressure solvent pump, an injector, a column, a detector, and a data recorder (Fig. 1.4).

Note: The high pressures referred to are of the order of 2000–6000 psi. Since we are working with liquids instead of gases, high pressures do not pose an explosion hazard. Leaks occur on overpressurizing; the worse problems to be expected are drips, streams, and puddles.

Solvent (mobile phase) from a solvent reservoir is pulled up the solvent inlet line into the pump head through a one-way check valve. Pressurized in

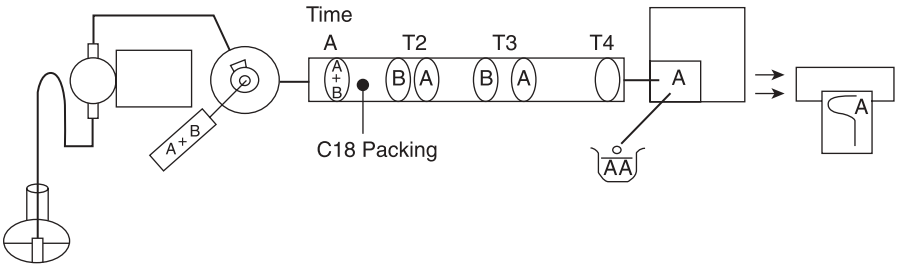

Figure 1.3 Separation model 2 (HPLC column).

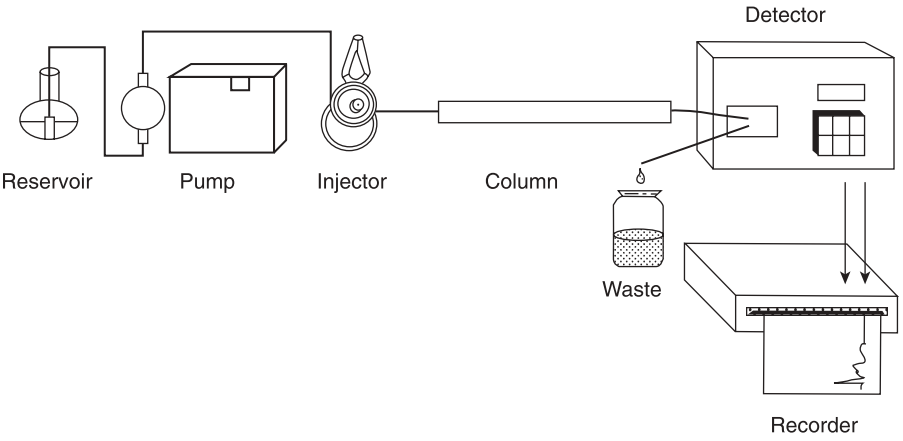

Figure 1.4 An isocratic HPLC system.

the pump head, the mobile phase is driven by the pump against the column back-pressure through a second check valve into the line leading to the sample injector. The pressurized mobile phase passes through the injector and into the column, where it equilibrates with the stationary phase and then exits to the detector flow cell and out to the waste collector.

The sample, dissolved in mobile phase or a similar solvent, is first loaded into the sample loop and then injected by turning a handle swinging the sample loop into the pressurized mobile phase stream. Fresh solvent pumped through the injector sample loop washes the sample onto the column head and down the column.

The separated bands in the effluent from the column pass through the column exit line into the detector flow cell. The detector reads concentration changes as changes in signal voltage. This change in voltage with time passed out to the recorder or computer over the signal cable and is traced on paper as a chromatogram, allowing fractions to be detected as rising and falling peaks.

There are always two outputs from a detector, one electrical and one liquid. The electrical signal is sent to the recorder for display and quantitation (analytical mode). The liquid flow from the detector flow cell consists of concentration bands in the mobile phase. The liquid output from nondestructive detectors can be collected and concentrated to recover the separated materials (preparative mode).

It is very important to remember that HPLC is both an analytical and a preparative tool. Often the preparative capabilities of the HPLC are overlooked. While normal analytical injections contain picogram to nanogram quantities, HPLCs have been used to separate as much as 1 lb in a single injection (obviously by a candidate for the *Guinness Book of World Records*). Typical preparative runs inject 1–3 g to purify standard samples.

To be effective, the detector must be capable of responding to concentration changes in all of the compounds of interest, with sensitivity sufficient to measure the component present in the smallest concentration. There are a variety of HPLC detectors. Not all detectors will see every component separated by the column. The most commonly used detector is the variable ultraviolet (UV) absorption detector, which seems to have the best combination of compound detectability and sensitivity. Generally, the more sensitive the detector, the more specific it is and the more compounds it will miss. Detectors can be used in series to gain more information while maintaining sensitivity for detection of minor components.

1.1.3 Use of Solvent Gradients

Solvent gradients are used to modify the separations achieved in the column. We could change the separation by changing the polarity of either the column or the mobile phase. Generally, it is easier, faster, and cheaper to change the character of the solvent.

The key to changing the separation is to change the difference in polarity between the column packing and the mobile phase. Making the solvent polarity more like the column polarity lets compounds elute more rapidly. Increasing the difference in polarities between column and mobile phase makes compounds stick tighter and come off later. The effects are more dramatic with compounds that have polarities similar to the column.

On a nonpolar column running in acetonitrile, we could switch to a more polar mobile phase, such as methanol, to make compounds retain longer and have more time to separate. We can achieve much the same effect by adding a known percentage of water, which is very polar, to our starting acetonitrile mobile phase (step gradient). We could also start with a mobile phase containing a large percentage of water to make nonpolar compounds stick tightly to the top of the column and then gradually increase the amount of acetonitrile to wash them off (solvent gradient). By changing either the initial amount of acetonitrile, the final amount of acetonitrile, or the rate of change of acetonitrile addition, we can modify the separation achieved. Separation of very complex mixtures can be carried out using solvent gradients. There are, however, penalties to be paid in using gradients. More costly equipment is required, solvent changes need to be done slowly enough to be reproducible, and the column must be re-equilibrated before making the next injection. Isocratic separations made with constant solvent compositions can generally be run in 5–15 min. True analytical gradients require run times of around 1 hr with about a 15-min re-equilibration. But some separations can only be made with a gradient. We will discuss gradient development in a later section.

1.1.4 Ranges of Compounds

Almost any compound that can be retained by a column can be separated by a column. HPLC separations have been achieved based on differences in polarity, size, shape, charge, specific affinity for a site, stereo, and optical isomerism. Columns exist to separate mixtures of small organic acid present in the Krebs cycle to mixtures of macromolecules such as antibody proteins and DNA restriction fragments. Fatty acids can be separated based on the number of carbons atoms in the chains or a combination of carbon number and degree of unsaturation. Electrochemical detectors exist that detect separations at the picogram range for rat brain catecholamines. Liquid crystal compounds are routinely purified commercially at 50 g per injection. The typical injection, however, is of 20 μL of solvent containing 10–50 ng of sample. Typical runs are made at 1–2 mL/min and take 5–15 min (isocratic) or 1 hr (gradient).

1.2 OTHER WAYS TO MAKE MY SEPARATION

Obvious there are many other analytical tools in the laboratory that could be used to make a specific separation. Other techniques may offer higher

sensitivity, less expensive equipment, different modes of separation, or faster and dirty tools for cleaning a sample before injection into the HPLC. Often, a difficult separation can only be achieved by combining these tools in a sequential analysis or purification. I'll try to summarize what I know about these tools, their strengths and drawbacks.

1.2.1 FPLC—Fast Protein Liquid Chromatography

FPLC is a close cousin of the HPLC optimized to run biological macromolecules on pressure-fragile agarose or polymeric monobead-based columns. It uses the same basic system components, but with inert fluid surfaces (i.e., Teflon, titanium, and glass), and is designed to operate at no more than 700 psi. Inert surfaces are necessary since many of the resolving buffers contain high concentrations of halide salts that attack and corrode stainless steel surfaces. Glass columns are available packed with a variety of microporous, high-resolution packings: size, partition, ion exchange, and affinity modes. A two-pump solvent gradient controller, injector valve, filter variable detector, and a fraction collector complete the usual system. The primary separation modes are strong anion exchange or size separation rather than reverse-phase partition as in HPLC.

FPLC advantages include excellent performance and lifetimes for the monobead columns, inert construction against the very high salt concentrations often used in protein chromatography, capability to run all columns types traditionally selected by protein chemist, availability of smart automated injection and solvent selection valves, and very simple system programming. Disadvantages include lack of capability to run high-pressure reverse phase columns, lack of a variable detector designed for the system, and lack of a true autosampler. HPLC components have been adapted to solve the first two problems, but have proved to be poor compromises. The automated valves can partially compensate for the lack of an autosampler.

1.2.2 LC—Traditional Liquid Chromatography

LC is the predecessor of HPLC. It uses slurry packed glass column filled with large diameter (35–60 μm) porous solid material. Materials to be separated are dissolved in solvent and applied directly to the column head. The mobile phase is placed in a reservoir above the column and gravity fed to the column to elute the sample bands. Occasionally, a stirred double-chamber reservoir is used to generate linear solvent gradients and a peristaltic pump is used to feed solvent to the column head. Packing materials generally made of silica gel, alumina, and agarose are available to allow separation by partition, adsorption, ion exchange, size, and affinity modes.

A useful LC modification is the quick clean-up column. The simplest of this is a capillary pipette plugged with glass wool and partially filled with packing material. The dry packed column is wetted with solvent, sample is applied, and